Ribosomal DNA contributes to global chromatin regulation

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The 35S ribosomal RNA genes (*rDNA*) are organized as repeated arrays in many organisms. Epigenetic regulation of transcription of the rRNA results in only a subset of copies being transcribed, making *rDNA* an important model for understanding epigenetic chromatin modification. We have created an allelic series of deletions within the *rDNA* array of the *Drosophila Y* chromosome that affect nucleolus size and morphology, but do not limit steady-state rRNA concentrations. These *rDNA* deletions result in reduced heterochromatin-induced gene silencing elsewhere in the genome, and the extent of the *rDNA* deletion correlates with the loss of silencing. Consistent with this, chromosomes isolated from strains mutated in genes required for proper heterochromatin formation have very small *rDNA* arrays, reinforcing the connection between heterochromatin and the *rDNA*. In wild-type cells, which undergo spontaneous natural *rDNA* loss, we observed the same correlation between loss of *rDNA* and loss of heterochromatin-induced silencing, showing that the volatility of *rDNA* arrays may epigenetically influence gene expression through normal development and differentiation. We propose that the *rDNA* contributes to a balance between heterochromatin and euchromatin in the nucleus, and alterations in *rDNA*—induced or natural—affect this balance.

Drosophila | epigenetics | heterochromatin | nucleolus | rDNA

Chromatin within the nucleus is divided into cytologically heterochromatic and euchromatic compartments (1). This division reflects very different functional influences on gene expression (2). Many genes adopt more "heterochromatin-like" features when inactivated, including cytological appearance and association with specific proteins or post-translational modifications. This has led to hypotheses that similar mechanisms regulate facultatively inactivated genes or chromosomes, constitutively heterochromatic regions of the genome, and developmentally repressed genes (3). Understanding the interplay between heterochromatin and euchromatin, then, is fundamental in understanding the control of epigenetic regulation of the genome.

Gene products involved in heterochromatin formation have been primarily identified by observing the effect of mutations on position effect variegation (PEV), which manifests as mosaic expression of a gene placed in a heterochromatic context. Many of these mutations act dominantly, thus the genes are thought to encode dose-sensitive components of heterochromatin (4). Equally important to models of heterochromatin formation is the observation that the amount of constitutive heterochromatin in the nucleus affects heterochromatin-induced PEV at unlinked genes (5). In this model, gene products act as a "source" of heterochromatin forming potential, and DNA sequences destined to be heterochromatic as a "sink." A balance is normally maintained between gene products and target DNA in the genome, although no proposed mechanism satisfactorily accounts for how this balance is maintained during division, determination, and differentiation. In comparison to our growing understanding of the protein components of heterochromatin, we have little understanding of the *cis*-acting components of heterochromatin. Experiments have shown that blocks of heterochromatin with different sequence composition differ in their ability to affect variegating gene expression, and polymorphisms on heterochromatic chromosomes can affect even non-variegating gene expression (6), but how these sequences differ in their ability to affect gene expression is not known. These observations led us to believe that understanding heterochromatin sequences will be necessary to understand the nature and regulation of chromatin in a developing cell.

We sought to investigate the role of a particular component of heterochromatin—the ribosomal DNA (*rDNA*)—on gene regulation. The *rDNA* is organized as a repeat array in most organisms (7), and expression of individual cistrons accounts for approximately 50% of total cellular transcription which provides rRNA for ribosomes. Sequences within the repeated *rDNA* nucleate the nucleolus (8), a subnuclear structure which has functions in addition to its role in ribosome biosynthesis. The *rDNA* and nucleolus have played a prominent role in evolving theories of aging, metabolism, cell differentiation, cell cycle control, cancer progression, and gene regulation (9–19). The *rDNA* is of particular interest in understanding heterochromatin because it is known to be regulated by epigenetic modification (20–24), is associated with both active and repressive protein modification (25, 26), can affect variegation at unlinked genes (27, 28), can itself induce variegation (29–31), and may change its size and regulation through the lifespan of an organism (32, 33). Few studies, however, have probed the connection between the *rDNA*, nucleolus, and heterochromatin formation in the nucleus.

We have developed a technique to create and measure the extent of specific allelic deletions within the *rDNA*, and measure the resulting effects on the amount of heterochromatin in the nucleus. We have found that deletions of the *rDNA* affect gene expression elsewhere in the genome as a result in decreased heterochromatic composition of the genome, in much the same manner as mutations in known protein heterochromatin components. This is despite negligible effects to translational capacity, suggesting that the nucleolus structure, rather than rRNA output, is important in regulating the heterochromatin. Correspondingly, we show that *rDNA* arrays isolated from mutants of known heterochromatin components are unusually small. We therefore propose that the *rDNA* contributes to a balance between heterochromatic and euchromatic compartments within the nucleus. Further, we show that natural loss of *rDNA* through development parallels loss of silencing of a variegating transgene, supporting our model that reduced *rDNA* copy number results in reduced heterochromatin-forming potential, and suggesting that natural differences in rates of *rDNA* loss may impact gene expression in developing cells. We discuss how this model provides an explanation for clonal inheritance of heterochromatin-induced gene silencing.

Results

Using methods developed in our laboratory, we created and characterized an allelic series of deletions within the *Y*-linked *rDNA* array of *Drosophila melanogaster*. We were able to recover unbiased deletions by generating and maintaining *rDNA* deletion chromosomes in the presence of *X* chromosomes that possessed full-length *rDNA* arrays. We could make the *Y*-linked *rDNA* arrays the sole source of *rDNA* in the organism (Fig. 1) and measured the size of

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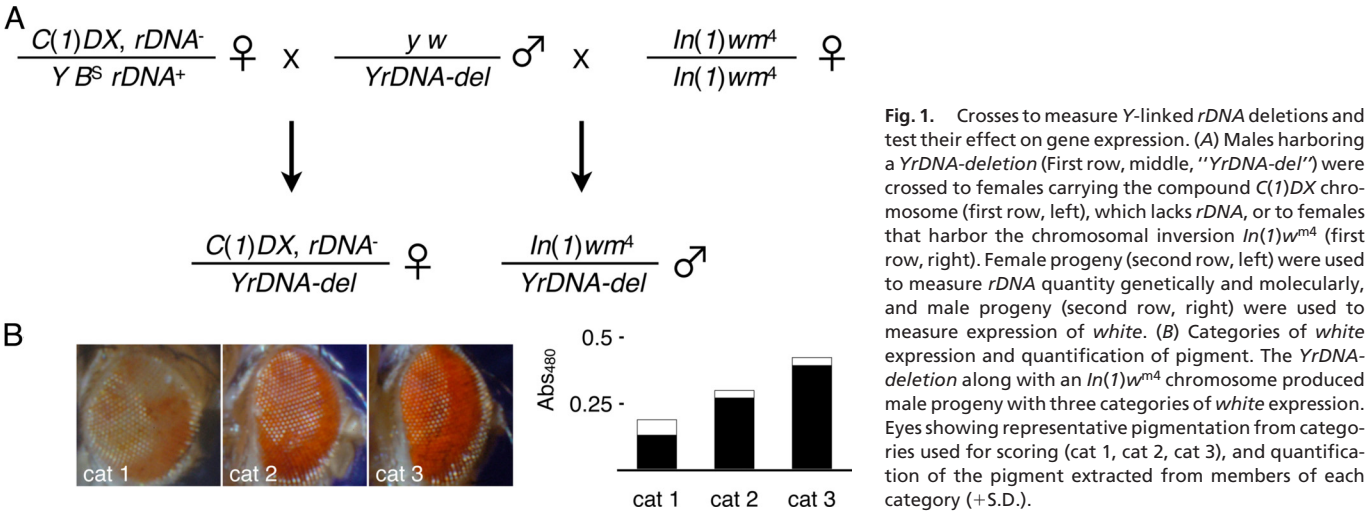

Fig. 1. Crosses to measure *Y*-linked *rDNA* deletions and test their effect on gene expression. (*A*) Males harboring a *YrDNA-deletion* (First row, middle, ''*YrDNA-del*'') were crossed to females carrying the compound *C(1)DX* chromosome (first row, left), which lacks *rDNA*, or to females that harbor the chromosomal inversion *In(1)w*^{m4} (first row, right). Female progeny (second row, left) were used to measure *rDNA* quantity genetically and molecularly, and male progeny (second row, right) were used to measure expression of *white*. (*B*) Categories of *white* expression and quantification of pigment. The *YrDNA-deletion* along with an *In(1)w*^{m4} chromosome produced male progeny with three categories of *white* expression. Eyes showing representative pigmentation from categories used for scoring (cat 1, cat 2, cat 3), and quantification of the pigment extracted from members of each category (+S.D.).

the deletions using genetic activity and real-time PCR. Based on the "bobbed" phenotype, which manifests as a result of limited translational capacity in protein-synthesis-intensive tissues (such as cuticular and bristle secreting cells), we divided deletions into two categories: "small deletions" limited for rRNA production and expressing a bobbed phenotype, and "large deletions" incapable of providing sufficient rRNA when the sole source of *rDNA* in the cell and expressing a bobbed-lethal phenotype. These categories were confirmed using real-time PCR to measure the number of *rDNA* cistrons in the array (34).

rDNA deletions were also tested for their effects on expression of the *white* gene of the well-studied *Inversion(1)-white-mottled-4* (*w*^{m4}) allele, which imposes heterochromatin-induced silencing (position effect variegation, or PEV) of the *white* gene. This genetic background effectively complements the *rDNA* deletions due to ample *rDNA* on the *X* chromosome. We tested 25 *rDNA* deletions and found that nine acted as weak suppressors of silencing, moderately reactivating *white* expression, and 16 had strong suppressor effects, reactivating *white* expression to nearly wild-type levels. These categories corresponded to small and large deletions, respectively, and when we aligned data for *rDNA* array size and *w*^{m4} expression, we saw a clear correlation between the size of the *rDNA* deletion and increased expression of *w*^{m4} (Fig. 2*A*, red bars). We confirmed the increased *w*^{m4} expression caused by *rDNA*-deleted chromosomes affected heterochromatin in general, and not just this particular allele of *white*, by testing effects of the *rDNA* deletions on two other variegating alleles.

White-mottled-4h is an inverted *X* chromosome with a different proximal (heterochromatic) breakpoint than *w*^{m4} (29), and deletions cause the same increase in expression of *w*^{m4h} (Fig. 2*A*, blue bars). If deleting the *rDNA* affects the nature or amount of heterochromatin in the nucleus, we expected that a silenced allele of a heterochromatic gene might show an opposite response to the deleted *Y*-linked *rDNA*. The *light* gene normally resides in the heterochromatin of chromosome *2*, and undergoes variegated gene silencing when translocated or inverted to euchromatin (35). Deletion of the *rDNA* showed an increase in *light* silencing relative to an undeleted *Y* (Fig. 2*B*), consistent with a shift in the balance between heterochromatin and euchromatin.

Together, the effects on silencing of *w*^{m4}, *w*^{m4h}, and *lt*^{var} support our hypothesis that deletions of the *rDNA* generally decrease the "heterochromatic" compartment in the nucleus. This experimental outcome is consistent with the dose- and environmental-sensitivity of heterochromatin-induced silencing (36), and in particular the work of Lloyd and colleagues which showed that silencing at one site within the genome affected the extent of silencing elsewhere, indicating a balance between heterochromatin and euchromatin (37).

Some short *rDNA* arrays can increase in size through meiotic magnification, resulting in heritable alterations in mean *rDNA* array size in a population (38, 39). The deletions we generated possess the ability to magnify at a rate of up to 15 copies per fly generation (34), which provided us the opportunity to confirm the correlation between expression and *rDNA* deletion. We observed expression in six strains as they magnified and simultaneously measured the quantity of *rDNA*. Expression decreased concordant with magnification in *rDNA* amount (Fig. 3).

Mutations in many genes involved in heterochromatin formation act dominantly, suggesting the gene products are dose-sensitive. Therefore, one possible cause of decreased heterochromatin in our deleted *Y*-linked *rDNA* arrays could be decreased translational capacity. We did not expect that to be the case because other studies have shown that approximately one hundred copies of *rDNA* are sufficient for viability (40, 41), and the flies in which the suppressed silencing was measured have approximately 400 copies on the *w*^{m4} chromosome alone. Nonetheless, to confirm that ample rRNA was provided by the *X*-linked *rDNA* array in our experiments, we isolated total RNA from adult flies of genotype *w*^{m4}*/YrDNA*-deletion, and confirmed that the rRNA encoded by the deleted *35S* cluster was not decreased in either small or large *rDNA* deletions (Fig. 4*A*), consistent with the presence of the wild-type *X*-linked *rDNA* array, the long half-life of these RNAs, and potential compensatory transcriptional regulation (42–44).

In contrast to final concentration of rRNA, the *rDNA* deletions do differ in nucleolar volume and morphology from wild-type strains. We used 3-D reconstruction of confocal stacks of whole mount salivary gland nucleoli to measure the volume of the fibrillary component of the nucleoli. Deleted *rDNA* arrays nucleated smaller nucleoli which frequently fragmented, appearing with ectopic small or micro nucleoli (Fig. 4 *B–E*). This fragmentation was not seen in any of our wild-type preparations and may be a manifestation of altered regulation or magnification of our deleted alleles. Similar alterations in nucleolar size, number, and morphology appear in some differentiated or cancerous cells (45).

Pimpinelli and colleagues showed mutations in *modulo*, a suppressor of variegation, interact genetically and cytologically with the *rDNA* (12), and Peng and Karpen showed that genes required for heterochromatin formation also had effects on the structure of the nucleolus, causing the formation of extrachromosomal circles and consequent supernumerary nucleoli (23). They hypothesized this phenotype to arise from disruption of the heterochromatic "closed" nature of the *rDNA*, and subsequent increase in intrachromosomal recombination. Consistent with this, we found that *Y* chromosomes isolated from stocks of *Su(var)3–9* and *Su(var)2–1* had *Y* chromosomes with small *rDNA* arrays (Fig. 5*A*), which expressed a bobbed

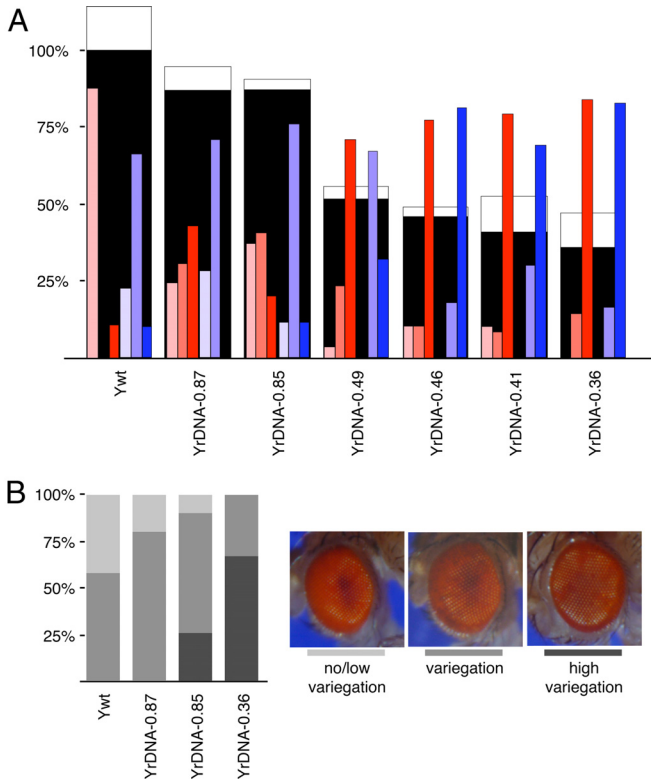

Fig. 2. rDNA deletions result in a loss of heterochromatin. (*A*) Inverse correlation between *rDNA* amount and *white* expression, determined one generation after the deletions were created. Black bars, mean amount of *rDNA* (+ S.D.), relative to the parental *Ywt* chromosome. Light, medium, and dark red represent the percentage of males found in each category of expression (Fig. 1). Light, medium, and dark blue represent similar categories of expression for the w^{m4h} allele. Chromosome names indicate the fraction of *rDNA* (relative to *Ywt*), as described in *Materials and Methods*. *P* values (Student's *t*-test) for significant differences: *YrDNA*-wt vs. *YrDNA*-0.87 ($P = 0.036$), *YrDNA*-wt vs. *YrDNA*-0.85 ($P = 0.025$), *YrDNA*-0.87 vs. *YrDNA*-0.85 ($P = 0.950$), *YrDNA*-0.85 vs. *YrDNA*-0.49 ($P < 0.001$), *YrDNA*-49 vs. *YrDNA*-0.46 ($P = 0.01$), *YrDNA*-0.46 vs. *YrDNA*-0.41 ($P = 0.23$), *YrDNA*-0.46 vs. *YrDNA*-0.36 ($P = 0.027$), *YrDNA*-41 vs. *YrDNA*-0.36 ($P = 0.36$). (*B*) Enhancement of $light^{x13}$/*light* variegation by three *YrDNA-deletion* chromosomes. Light gray represents wild-type eyes, medium gray represents eyes with evident variegation (dark patches), and dark gray represents entirely dark eyes (extreme variegation). Examples of wild-type pigmentation (top), variegation (left) and extreme variegation (right) are shown.

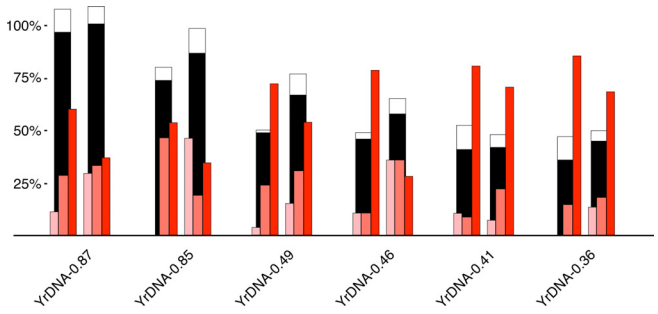

Fig. 3. rDNA magnification reverts the phenotype. Subsequent generations of the *rDNA* deletion alleles in Fig. 2*A*. Black bars indicate mean amount of *rDNA* (+ S.D.), relative to the parental *Ywt* chromosome. Light, medium, and dark red represent the percentage of males found in each category of expression for w^{m4}. Two generations are shown for each chromosome, showing *rDNA* magnification is concomitant with loss of silencing. *P* values (Student's *t*-test) for each magnification: *YrDNA*-0.87 generations ($P = 0.393$), *YrDNA*-0.85 ($P = 0.016$), *YrDNA*-0.49 ($P < 0.001$), *YrDNA*-0.46 ($P = 0.002$), *YrDNA*-0.41 ($P = 0.818$), *YrDNA*-0.36 ($P = 0.051$).

phenotype when made sole source of *rDNA* (Fig. 5 *B* and *D*) and showed incompletely penetrant, low expressivity bobbed phenotype in a stock which contains both *X*- and *Y*-linked *rDNA* arrays (Fig. 5 *C* and *E*). Our deletions show that reduced *rDNA* arrays act as suppressors of heterochromatic silencing and result in altered nucleolar morphology, just as mutations in these two genic suppressors of variegation result in short *rDNA* arrays which also have altered nucleolar morphology (23).

The prevailing view of heterochromatin-induced silencing is that stochastic decisions to become inactive ("heterochromatic") or remain active ("euchromatic") occur at a gene found near a new heterochromatin/euchromatin boundary. This gives rise to stable decisions in the "deciding" and any progeny cells, resulting in the familiar patches of expressing cells and non-expressing cells which reflect cell lineage (46). This view predicts that every set of genes linked to a heterochromatic/euchromatic junction is independent, and that decisions made at one junction influence closely-linked genes, but not genes near other junctions. Our view that the *rDNA* influences the genomic balance of heterochromatin and euchromatin predicts that the extent of silencing will be a cellular phenomenon rather than a gene-locus phenomenon, and, more specifically, that the extent of silencing will be correlated with the amount of *rDNA* in a cell.

The *rDNA* undergoes somatic recombination (32, 33, 42, 43, 47), and we wondered if natural *rDNA* fluctuations might occur and contribute to expression patterns of a variegating gene in wild-type cells. We created a *Y*-linked variegating GFP transgene that has a large variance in level of expression, allowing us to dissect patches of expressing and non-expressing tissue from third instar larvae. Paired bilaterally-symmetrical optic lobes that showed different levels of expression, or fragments of one lobe with local differences in GFP expression, were separately used to measure *rDNA* amount in the cells. We found that those brain fragments with higher GFP expression had less *rDNA* than non-expressing tissue from the same brain (Fig. 6 *A–F*), in contrast to different patches of tissue with no GFP expression, which have similar quantities of *rDNA* (Fig. 6 *G* and *H*). This shows that natural decrease of *rDNA* copy number may act the same as our induced deletions, and affect gene expression by decreasing the amount of heterochromatin-induced silencing.

Discussion

We have shown that deletions within the *Y*-linked ribosomal DNA (*rDNA*) arrays of *Drosophila* reduce the extent of heterochromatin-induced gene silencing at unlinked genes. We showed that multiple genes are affected, including those that are silenced by heterochromatin and those that are activated by heterochromatin. Taken together, our results suggest that deletions within the *rDNA* shift a balance of heterochromatin and euchromatin to a more euchromatic nature of the nucleus. We envision that sufficiently short arrays create a nuclear milieu more permissive for gene expression, while those arrays of longer size do not. Since our *rDNA* deletions cause a loss of heterochromatin, they act like classical mutations in *Su*(*var*) genes. We see that mutations in two known heterochromatin components [*Su*(*var*)*3–9* and *Su*(*var*)*2–1*] possess *rDNA* arrays much shorter than those found in wild-type flies. Therefore, it is possible that some of the loss of silencing in these *Su*(*var*) mutations may be a result of first reducing the *rDNA*. The linkage between the histone methyltransferase encoded by *Su*(*var*)*3–9* is well-established, and we do not see a reason to doubt that *Su*(*var*)*3–9* works at the site of heterochromatin formation to suppress heterochromatin-induced silencing, but our results suggest that *Su*(*var*)*3–9* may additionally have an indirect role, through the *rDNA*, in suppressing gene silencing.

How the cell monitors *rDNA* length (or activity) is not yet clear—inactive cistrons may bind to repressive factors (e.g., com-

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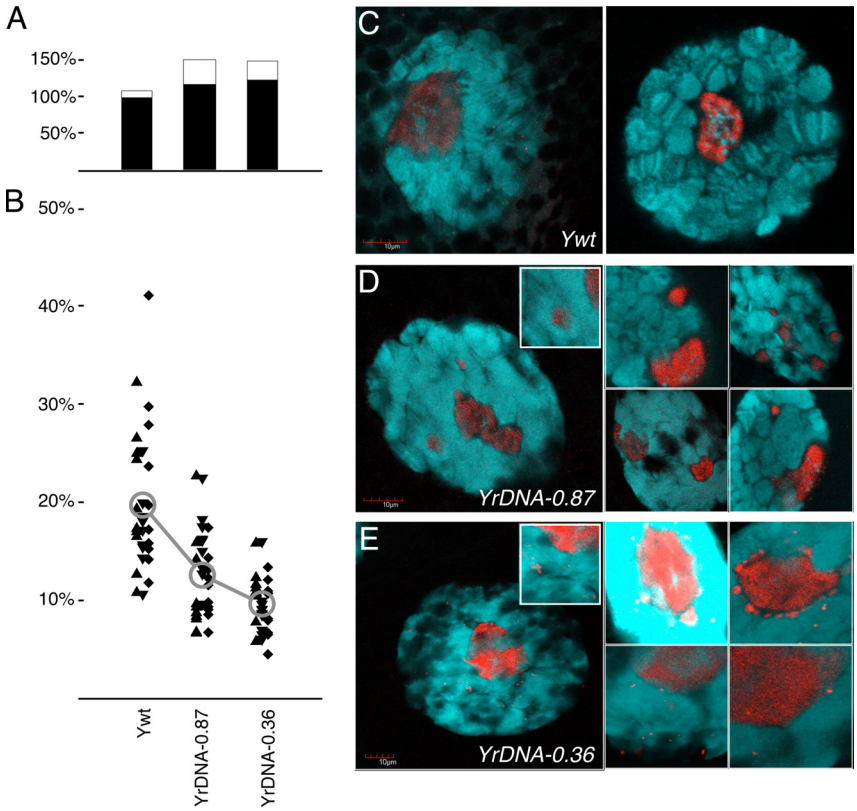

Fig. 4. Comparison between rRNA concentration and nucleolar volume and morphology between wild-type and *YrDNA*-deletion flies. (*A*) Comparison of rRNA between adults of genotype w^{m4}/*YrDNA*-deletion, relative to *Ywt* (which is defined as 100%), black bars, mean (±S.D.). Values seem elevated, but not significantly so (Student's *t*-test): *Ywt* vs. *YrDNA*-0.87 ($P = 0.304$), *Ywt* vs. *YrDNA*-0.87 ($P = 0.111$). (*B*) Quantification of nucleolar volume as fraction of total nuclear volume from 3-D reconstructed salivary gland nuclei of wild-type (*Ywt*), *YrDNA-0.87*, and *YrDNA-0.36* [$n = 30$ nuclei for each genotype, all data are shown, gray circles indicate the mean of combined data sets, Student's *t*-test: *Ywt vs. YrDNA*-0.87 ($P < 0.001$), *YrDNA*-0.87 vs. *YrDNA*-0.36 ($P = 0.003$)]. (*C–E*) confocal images of nuclei from *C(1)DX/Ywt*, *YrDNA-0.87*, and *YrDNA-0.36* processed for immunofluorescence to fibrillarin (red) and stained with DAPI to reveal DNA (blue). Insets for *YrDNA-0.87* and *YrDNA-0.36* are increased magnification showing mininucleoli and micronucleoli in respective chromosome preparations. Panels show range of mininucleolar and micronucleolar phenotypes of *YrDNA-deletion* chromosomes.

ponents of chromatin remodeling complexes) and deplete them from the rest of the genome, may generate a diffusible activating signal, may alter a balance between RNA polymerase I and RNA polymerase II transcripts, or a balance with other compartments or sequences (48–51). Others have noted the opposite effect—increased silencing with decreased *X*-linked *rDNA* arrays of males (27, 28). Whether the *X*-linked and *Y*-linked arrays are fundamentally different remains a question, although there are clear differences in sequence and epigenetic regulation of these arrays (20, 24). This raises the intriguing possibility that these two arrays may together establish a homeostasis of chromatin while jointly assuring sufficient translational capacity to the cell. Independent regulation (43, 52) could thus account for loss or underrepresentation of *rDNA* while simultaneously allowing for maintenance of translational capacity and heterochromatin-forming potential.

That the *rDNA* affects heterochromatin is particularly intriguing, since many repeated DNA arrays, including the *rDNA*, may shrink during development. Natural loss, then, and the resultant shift in heterochromatin/euchromatin balance may provide a simple explanation for the progressive loss of heterochromatic silencing in differentiating cells (46) and an explanation for why some epigenetic states are clonally inherited. We envision that cells initially contain large *rDNA* arrays, which permits heterochromatin formation. As a cell divides and approaches terminal differentiation, *rDNA* is lost and this milieu changes. Loss could occur through recombination or damage leading to extrachromosomal acentric *rDNA* circles (33) or through unequal sister chromatid exchange (39, 47). Cells which lose *rDNA* early in their lineage pass a threshold, lose some heterochromatin forming potential, and allow activation of silenced genes. Other cells, however, may have a slower rate of *rDNA* loss, do not cross the threshold, and thus remain silenced. Mutations which affect heterochromatin formation and nucleolar structure (23) may contribute to expression by increasing the rate at which *rDNA* is lost. Since *rDNA* loss would be largely irreversible, a cell which loses sufficient *rDNA* to compromise heterochromatin forming potential would give rise to progeny cells equally compromised, resulting in the familiar clonal patches of variegating gene expression.

We do not think that the effects we see here are unique to *Drosophila*. Heritable genetic modification has been mapped to variation in the *rDNA* of plants, and may also be responsible for somaclonal variation in cloned plant genotrophs (53–56). Alteration of nucleolar appearance during cancer progression, alterations in *rDNA* content in aging cells, and stress responses mediated through nucleolar *sir2* gene family members (11, 49), may underlie some aspects of these complex phenotypes in other organisms. Indeed, the complexities of these phenotypes may be compounded by the profound variation that exists within and between the *rDNA* loci of humans (57).

Somatic elimination of repeated DNAs is not unique to flies (58), nor is it restricted to the *rDNA* (32, 33); the extent to which it affects other repeated heterochromatic DNA is unknown (59). Lemos and colleagues recently showed polymorphisms of heterochromatic *Y* chromosomes, but did not map the source of those polymorphisms (6). Although our results establish a causal link between *rDNA* and gene expression, we also consider that other sequences, less easily manipulated or measured than the *rDNA*, might also contribute to a dynamic balance between heterochromatin and euchromatin during determination and differentiation. In a simple source-sink model of heterochromatin regulation, all heterochromatin is treated as equally potent in sequestering or binding heterochromatic proteins. Our results are consistent with a balance between heterochromatin-binding proteins and DNA destined to be packaged as heterochromatin, however our results demonstrate that the *rDNA* is at least one repeat that can alter the balance between source and sink dramatically. It will be exciting to discover how the dynamic constitution and structure of a genome might influence cell fate or the expressivity of complex phenotypes.

Materials and Methods

Fly Strains and Nomenclature. *YrDNA* deletion strains are described in Paredes and Maggert (34), but for ease have been given different names here, which

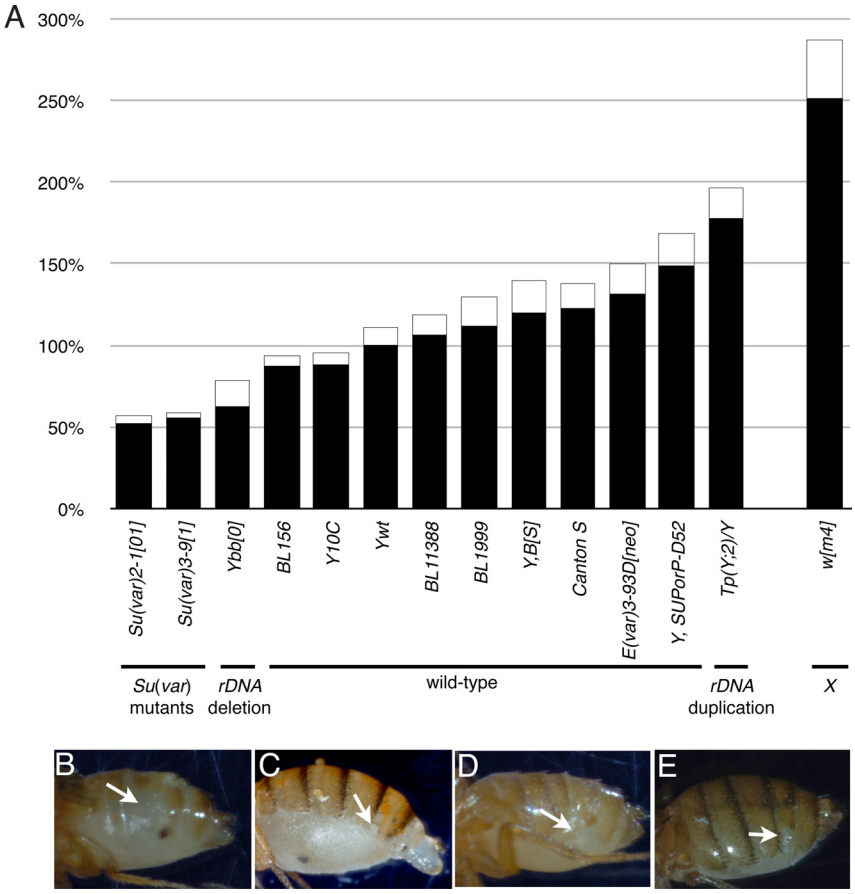

Fig. 5. Mutants of known heterochromatin proteins have small *rDNA* arrays. (*A*) *Y*-linked *rDNA* arrays were genetically isolated (as in the cross in Fig. 1*A*) from thirteen strains, including two known suppressors of variegation [*Su*(*var*)*2–1* and *Su*(*var*)*3–9*]. Real-time PCR quantification of *rDNA* array size is shown, black bars indicate mean amount of *rDNA* (+S.D.). *Ywt* is the undeleted wild-type chromosome used in this study. The rest of the chromosomes are described at http://flybase.bio.indiana.edu/. (*B*) *C*(*1*)*DX*/*Y* female whose *Y* chromosome was taken from the *Su*(*var*)*2–1*^{01} stock, showing severe etching bobbed phenotype (arrow). (*C*) *X*/*X* female from the *Su*(*var*)*2–1*^{01} stock, showing mild etching phenotype (arrow). (*D*) *C*(*1*)*DX*/*Y* female whose *Y* chromosome was taken from the *Su*(*var*)*3–9*^{1} stock, arrow shows moderate etching bobbed phenotype. (*E*) *X*/*X* female from the *Su*(*var*)*3–9*^{1} stock, arrow shows mild etching bobbed phenotype.

indicate the fraction of *rDNA* relative to the undeleted parental chromosome (*Ywt*). *Ywt* is y+*Y10B*, *YrDNA-0.87* is y^+*Y10B*, *YrDNA*$^{bb-465}$, *rDNA-0.85* is y^+*Y10B*, *YrDNA*$^{bb-76}$, *YrDNA-0.49* is y^+*Y10B*, *rDNA*$^{l-481}$, *YrDNA-0.46* is y^+*Y10B*, *YrDNA*$^{l-498}$, *rDNA-0.41* is y^+*Y10B*, *YrDNA*$^{l-510}$, and *YrDNA-0.36* is y^+*Y10B*, *rDNA*$^{l-473}$. *C*(*1*)*DX* is *C*(*1*)*DX*, y^1 f^1 *rDNA*^{0}, the wild-type *X* chromosome is y^1 w^{67c23}, *white-mottled* stocks are *In*(*1*)w^{m4} or *In*(*1*)w^{m4h}, *light-variegator* stock is *lt*^{x13}/*SM1*, *Cy lt*. Deleted *Y* chromosome-bearing males were backcrossed every generation to an isogenic stock. The fly strain variegating for green fluorescence protein, *Y10C*, is y^+*Y*, *rDNA*$^+$, *P*{*X97*, *ubiq-GFP*, w^+}*10C*, generated using *FLP*/*FRT*-mediated replacement (60) of a *GFP*$^{S65T.Ubi-p63E}$ transgene (cloned from y^1 *w**; *In*(*2LR*)*Gla*, *wg*$^{Gla-1}$ *Bc*^{1}/*CyO*, *P*{$w^{+mW.hs=Ubi-GFP.S65T}$}*PAD1*) at the *Y10B P*-element insertion site (34). In Fig. 5, the flies are 156: *al*^{1} *dp*^{ov1} *b*^{1} *pr*^{1} *c*^{1} *px*^{1} *sp*^{1}, 11388: *cn*^{1} *P*{$ry^{+t7.2=PZ}$}*AGO1*^{04845}/*CyO*; *ry*^{506}, 1999: *C*(*1*;*Y*)*6*, w^{1118}.

Dissection. Larvae were raised on standard cornmeal molasses fly food supplemented with baker's yeast and raised at 18 °C. Salivary glands or brains from wandering third instar larvae where dissected in PBS. Tissues destined for immunofluorescence were processed immediately. Tissues destined for real-time PCR were frozen at −70 °C.

Immunofluorescence/Confocal Microscopy. For immunofluorescence, salivary glands were washed in PBT (PBS supplemented with 0.1% Tween-80), blocked for 2 h in PBT with 10% BSA, and incubated with antibodies overnight at 4 °C in PBT supplemented with 1% BSA and 500 mM NaCl. Mouse anti-fibrillarin antibody (Abcam) was used at a 1:200 dilution, and goat anti-mouse conjugated to TRITC (Jackson ImmunoResearch Laboratories) was used at 1:200 as secondary antibody. Confocal fluorescent images were obtained on a Olympus FV1000 confocal

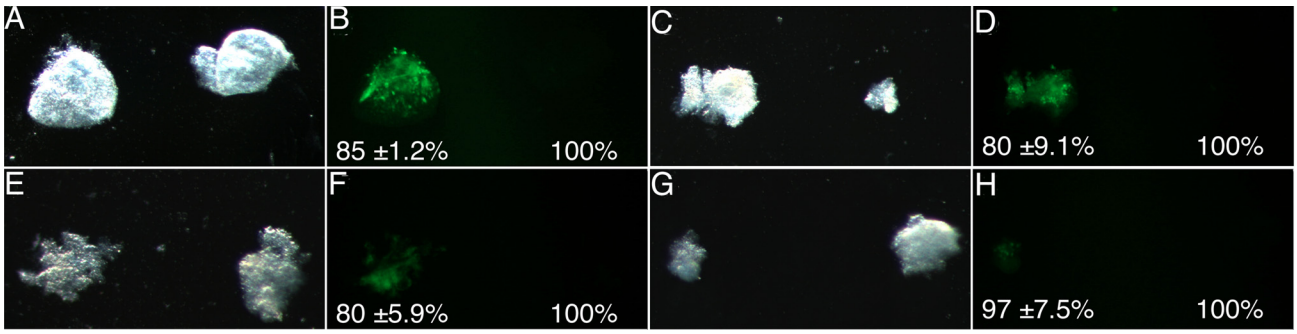

Fig. 6. Natural underrepresentation of *rDNA* correlates with gene expression in a *wild-type* individual. *rDNA* quantification of dissected brain tissue with different levels of variegating GFP expression. (*A*, *C*, *E*, and *G*) light microscopy of fragments of dissected optic lobes. (*B*, *D*, *F*, and *H*) GFP expression from dissected tissue. Values indicate *rDNA* quantified in the GFP-expressing tissue, relative to the *rDNA* in the non-expressing tissue (+ S.E.M.), which was defined as 100%. *P* values (Student's *t*-test) for each set: *A* and *B* ($P = 0.001$), *C* and *D* ($P = 0.113$), *E* and *F* ($P = 0.041$), *G* and *H* ($P = 0.655$). Four of eight experiments are shown; all eight showed the same trend of decreased *rDNA* in GFP-expressing tissue relative to non-expressing tissue (for those data not presented, GFP-expressing patches had relative *rDNA* amounts of 85.0 ± 9.9% (S.E.M.), 87.3 ± 4.4%, and 84.7 ± 9.3%, while patches with no difference in GFP expression had a relative *rDNA* amount of 102.0 ± 5.6%).

microscope with a 100× immersion oil objective. Sequential excitation with lasers was done at 405 nm and 543 nm to observe DAPI staining and rhodamine, respectively, and were analyzed with FV10-ASW 1.7 Viewer software. Three dimensional reconstruction of nucleoli and nucleus was done using ImageJ with the LOCI and Voxel-Counter plug-ins. Nucleolus volume was determined relative to the total nucleus. Ten nucleoli were analyzed in each of three different salivary glands for each fly line analyzed.

DNA Preparations. DNA was extracted from single larval or adult flies as described in Paredes and Maggert (34). DNA was quantified using a Nanodrop and diluted to 10 ng/μL. Triplicate real-time PCR reactions were performed with 10 ng template. For dissected brains, frozen tissue was sonicated in 200 μL PBS using a Misonix XL-2000 with three 10-s pulses and 20-s intervals. One microliter from the sonicated sample was used in each of triplicate real-time PCR reactions. Primers, controls, and data analyses are described in Paredes and Maggert (34).

RNA Analyses. RNA was extracted according to Bogart and Andrews (61). Pupae were *C(1)DX/YrDNA-deletion*, identified using the *Y*-linked *yellow*$^+$ gene of *Ywt* (62), and adult flies were *w*^{m4}*/YrDNA-deletion*. RNA was electrophoretically separated at 100 V for 215 min in 1.5% agarose with running buffer 400 mM Mops (3-morpholinopropanesulfonic acid, 3-(N-morpholino)propanesulfonic acid), pH 7.0, 100 mM sodium acetate, and 10 mM EDTA (EDTA) supplemented with 18% formaldehyde. RNA was stained with ethidium bromide and quantified relative to tRNA using a Typhoon TRIO Variable Mode Imager (GE Healthcare) running ImageQuant 5.2. RNA was isolated from five pools of 10 flies each for comparison.

Pigment Extraction. Fly heads were removed by banging frozen flies, and incubated in 8% NaOH, 66% ethanol (50 μL per head) in the dark for 24 h at 37 °C. Pigment quantification was done using a BioRad SmartSpec3000 spectrophotometer at 320 nm (63) and 480 nm (64).

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